Characterization of a Caffeine-Resistant Mutant of *Aspergillus parasiticus:* Role of Amino Acid Metabolism

ABSTRACT

Aspergillus parasiticus BCR1, a caffeine-resistant mutant of *A. parasiticus* NRRL 2999, produced abundant amounts of aflatoxins (AF) in yeast extract-sucrose broth only when the medium was supplemented with caffeine. However, little AF production occurred in glucose-mineral salts medium (GMS) regardless of the level of caffeine supplementation. Caffeine-dependent AF production was restored if GMS were fortified with peptone or some other source of amino acids. Subsequent studies indicated that this effect could be achieved by supplementing GMS with specific amino acids, particularly proline, alanine, methionine, arginine or asparagine. Restoration of caffeine-dependent AF synthesis did not occur when GMS was supplemented with purine bases or nucleotides. The results indicated that caffeine-dependent AF production in BCR1 was dependent on amino acid catabolism.

INTRODUCTION

CAFFEINE (1,3,7-trimethylxanthine) is a naturally occurring inhibitor of *Aspergillus flavus* and *Aspergillus parasiticus* that helps protect tropical commodities such as coffee and cocoa beans from contamination by aflatoxins (Buchanan and Fletcher, 1978; Nartowicz et al., 1979; Lenovich, 1981; Buchanan et al., 1983; Betancourt and Frank, 1983; Buchanan and Lewis, 1984a; Durakovic et al., 1985a,b). Recent investigations (Buchanan et al., 1987) established that it is possible to isolate mutant strains of aflatoxigenic *A. parasiticus* that can grow in the presence of elevated levels of caffeine. Interestingly, these caffeine-resistant mutants only produced abundant amounts of aflatoxins on yeast extract-sucrose medium when it contained caffeine at levels that inhibit the wild type strain. As part of an effort to characterize further the caffeine-dependent nature of aflatoxin synthesis in caffeine-resistant strains of *A. parasiticus,* additional studies were undertaken using a more chemically defined medium. The current report summarizes those studies and identifies an apparent relationship between caffeine-dependent aflatoxin synthesis and amino acid metabolism.

MATERIALS & METHODS

Microorganism

Aspergillus parasiticus BCR1, a caffeine-resistant mutant derived from *Aspergillus parasiticus* NRRL 2999 (Buchanan et al., 1987), was used throughout the study. Stock cultures were grown until well sporulated on slants of potato-dextrose agar (Difco) supplemented with 2 mg/mL caffeine and then stored at 4°C. Spore suspensions were prepared as described previously (Buchanan et al., 1987) and diluted to contain 10^6 conidia/mL.

Media

Yeast extract-sucrose medium (YES) (Davis et al., 1966) consisted of 60g sucrose, 20g yeast extract (Difco), and 1L water. Glucose-mineral salts medium (GMS) (Buchanan and Lewis, 1984b) consisted of 60g glucose, 10g KH_2PO_4, 4g $(NH_4)_2SO_4$, 2g $MgSO_4$, 1 mL trace metal stock solution (Buchanan et al., 1983) and 1L water. Unless otherwise stated, the medium was adjusted to pH 6.0 using a 50% NaOH solution. Caffeine supplements were added prior to sterilization by autoclaving. Other supplements were added to the media either before or after (as filter-sterilized solutions) autoclaving, depending on their relative heat stability.

Culture techniques

Media were dispensed in 25 mL portions to 125 mL Erlenmeyer flasks, which were than capped with foam plugs and autoclaved for 15 min at 121°C. The flasks then received 0.5 ml spore suspension to achieve an inoculum of 2×10^4 conidia/mL. After gentle mixing, all flasks were incubated without agitation at 28°C. At specified times, replicate cultures were analyzed for pH, aflatoxin production and mycelium dry weight.

Analyses

After determining the pH of the medium using a pH meter equipped with a combination electrode, each culture (mycelium + medium) was extracted three times with 20 mL $CHCl_3$. The three extracts for each culture were pooled, concentrated on a rotary evaporator, transferred to a vial, evaporated to dryness under N_2 and redissolved in 1.0 mL $CHCl_3$. Aliquots were then spotted on thin-layer chromatography plates (series 7000 silica gel, 20 × 20 cm, Baker) along with aflatoxin standards and developed with chloroform/acetone/water (93:7:1). Aflatoxins (B_1 + B_2 + G_1 + G_2) were subsequently quantified using a fluorodensitometer (model CS-930, Shimadzu Corp., Kyoto, Japan). When needed, blue-fluorescent pyrazines and other interfering compounds (Buchanan and Houston, 1982) were eliminated by either pre-elution or post-elution with benzene/acetic acid (95:5) or anhydrous diethyl ether, respectively. Mycelium dry weights were determined gravimetrically by drying the extracted mycelia for 24 hr at 85°C.

RESULTS

THE RATE OF GROWTH of *A. parasiticus* BCR1 in YES decreased with increasing caffeine concentrations (Table 1); however, the extent of growth was equivalent with caffeine concentrations up to 4 mg/mL. Aflatoxins accumulated to only relatively low concentrations in YES without caffeine, with increasing amounts of aflatoxins occurring in response to increasing concentrations of caffeine up to 4 mg/mL. No aflatoxin production was detected in the 8 mg/mL cultures. In GMS, the general pattern of response to caffeine in regard to the rate and extent of growth of BCR1 was similar to that observed with YES. However, the caffeine-dependent production of aflatoxins observed in the YES cultures did not occur in the GMS cultures, with only low levels of aflatoxin accumulation being detected at all caffeine concentrations.

The differential response for aflatoxin productions between the nutritionally minimal GMS and the nutritionally more complete YES suggested that the caffeine-dependent synthesis of aflatoxins by BCR1 was dependent on a nutrient present in YES but absent from GMS. This was tested by supplementing GMS with yeast extract (Table 2). Since one of the primary

Table 1—Growth and aflatoxin production by Aspergillus parasiticus *BCR1 when cultured in GMS and YES*[a]

Medium	Caffeine (mg/mL)	Day	Mycelium dry weight (mg)	pH[b]	Aflatoxin/Culture (μg)	Aflatoxin/Mycelium (ng/mg)
YES	0	3	148(4)	5.17	0.51 (0.12)	3.5
		6	210(12)	7.11	0.34 (0.07)	1.6
		9	166(10)	8.28	3.16 (0.48)	19.0
	1	3	65(8)	5.57	0.60 (0.10)	9.2
		6	226(6)	6.06	6.16 (3.55)	27.3
		9	166(5)	8.27	10.61 (2.37)	63.9
	2	3	30(2)	5.91	1.74 (0.16)	57.4
		6	231(2)	5.64	91.67 (6.30)	397.2
		9	159(1)	8.24	82.26 (1.25)	517.4
	4	3	11(3)	6.27	1.68 (0.88)	151.4
		6	131(4)	5.59	122.93 (7.66)	938.4
		9	211(12)	7.47	265.83(31.22)	1259.9
	8	3	5(1)	6.44	0.00	0.0
		6	16(1)	6.31	0.00	0.0
		9	67(10)	6.35	0.00	0.0
GMS	0	3	11(1)	4.86	0.03 (0.01)	2.8
		6	131(10)	2.61	1.98 (1.91)	15.1
		9	127(7)	2.62	0.01 (0.01)	0.1
	1	3	10(1)	4.84	0.01 (0.01)	1.0
		6	115(3)	2.59	0.05 (0.01)	0.4
		9	143(1)	2.54	0.02 (0.01)	0.1
	2	3	9(1)	5.06	0.00	0.0
		6	124(17)	2.62	0.13 (0.02)	1.1
		9	147(7)	2.54	0.04 (0.01)	0.3
	4	3	6(1)	5.34	0.00	0.0
		6	74(2)	2.77	0.08 (0.01)	1.1
		9	260(26)	2.53	0.00	0.0
	8	3	6(1)	5.40	0.00	0.0
		6	13(2)	4.87	0.05 (0.05)	3.8
		9	130(12)	2.67	0.00	0.0

[a] Values = $\bar{x}(\pm SEM)$, n = 3 replicate cultures.
[b] Initial pH = 6.0.

Table 2—Effect of supplementation with yeast extract or peptone on aflatoxin production by A. parasiticus *BCRI cultured for 14 days in GMS with and without caffeine*[a]

Supplement[b]	Caffeine (mg/mL)	Mycelium dry weight (mg)	pH[c]	Aflatoxin/Culture (μg)	Aflatoxin/Mycelium (ng/mg)
None	0	169(2)	2.68	0.25(0.04)	1.5
	4	176(6)	2.79	0.79(0.45)	4.5
Yeast Extract	0	216(3)	7.15	0.98(0.12)	4.5
	4	160(6)	7.15	11.52(2.65)	72.1
Peptone	0	226(9)	7.66	0.01(0.01)	0.1
	4	153(2)	7.64	7.64(2.51)	50.0

[a] Values = $\bar{x}(\pm SEM)$, n = 3 replicate cultures.
[b] Supplements added at level of 20 mg/mL.
[c] Initial pH = 6.5.

nutritional functions of yeast extract is as a source of amino acids, an additional set of cultures was examined where GMS was supplemented with peptone (Table 2). In both cases, supplementation with these components led to a restoration of caffeine-dependent aflatoxin production, indicating that aflatoxin synthesis in BCR1 was dependent on the mold having sufficient sources of both caffeine and amino acids. When similar supplementation studies were performed using various purines (Table 3), there was little, if any, restoration of caffeine-dependent aflatoxin accumulation, indicating that this class of compounds was not involved in controlling toxin synthesis in BCR1.

The amount of aflatoxins produced in caffeine-containing GMS was dependent on the level of peptone supplementation (Table 4). An apparent threshold supplementation level of 20 mg/mL peptone was needed to restore caffeine-dependent aflatoxin production, with further increases in peptone levels resulting in increased aflatoxin accumulation. The observed differences in aflatoxin production did not appear due to changes in the rate or extent of growth resulting from increased levels of peptone.

Supplementation of GMS with individual amino acids indicated that some, but not all amino acids, restored caffeine-dependent aflatoxin synthesis (Table 5). Restoration of toxin production was particularly evident with alanine, arginine, asparagine, methionine and proline. Alternatively, aspartic acid, glutamine, glycine and serine had little, if any, effect. A few of the amino acids (e.g., proline) also appeared to stimulate toxin accumulation in the absence of caffeine; however, the degree of stimulation was small in comparision to that noted in the caffeine-containing cultures. In the absence of caffeine, most amino acids increased growth as compared to the unsupplemented controls. This general response was not observed with the caffeine-containing cultures. No general relationship between changes in mycelium dry weights and restoration of caffeine-dependent aflatoxin production was evident.

DISCUSSION

PREVIOUS INVESTIGATIONS (Buchanan et al., 1987) indicated that acquisition of caffeine-resistance in *A. parasiticus* was accompanied by an alteration in aflatoxin synthesis such that in YES abundant amounts of the toxins were only produced in the presence of caffeine. Sporulation by the caffeine-resistant isolates on YES was also caffeine-dependent, suggesting that the mutation did not involve a locus directly as-

Table 3—Effect of purine bases, nucleosides and nucleotides on aflatoxin production by Aspergillus parasiticus *BCRI cultured for 10 days in GMS with and without caffeine*[a]

Purine supplement[b]	Caffeine (mg/mL)	Mycelium dry wt (mg)	pH[c]	Aflatoxin Culture (μg)
None	0	158(4)	2.73	0.02(0.02)
	4	194(6)	2.54	0.40(0.11)
Adenine	0	206(10)	2.69	0.00
	4	209(3)	2.60	1.38(0.36)
Adenosine	0	185(11)	2.65	0.00
	4	211(1)	2.73	0.04(0.02)
AMP	0	182(10)	2.80	0.00
	4	193(1)	2.77	0.03(0.02)
Guanine	0	187(10)	2.73	0.00
	4	196(10)	2.51	1.55(0.21)
Guanosine	0	179(5)	2.78	0.00
	4	192(10)	2.61	0.29(0.29)
GMP	0	189(4)	3.14	0.00
	4	207(3)	2.69	0.02(0.02)

[a] Values = $\bar{x}(\pm SEM)$, n = 3 replicate cultures except no-supplement controls where n = 9 replicate cultures.
[b] 5 mM.
[c] Initial pH = 6.0.

Table 4—Effect of peptone supplementation level on aflatoxin production by A. parasiticus *BCRI in GMS medium*[a]

Peptone level (mg/mL)	Caffeine (mg/mL)	Day	Mycelium dry wt (mg)	pH[b]	Aflatoxin Culture (μg)	Aflatoxin Mycelium (ng/mg)
0	0	7	158(12)	2.87	0.00	0.0
		14	128(5)	2.73	0.26(0.13)	2.0
	4	7	75(16)	3.55	0.42(0.08)	5.6
		14	189(3)	2.46	0.00	0.0
10	0	7	113(7)	2.81	0.00	0.0
		14	197(7)	2.47	1.55(0.12)	7.9
	4	7	168(6)	2.88	0.03(0.03)	0.2
		14	152(9)	2.69	0.51(0.51)	3.7
20	0	7	180(12)	2.72	0.00	0.0
		14	167(6)	2.57	0.00	0.0
	4	7	113(14)	3.09	2.63(1.57)	23.2
		14	167(5)	2.72	6.27(0.64)	37.6
40	0	7	195(2)	2.78	0.00	0.0
		14	188(3)	2.63	0.00	0.0
	4	7	141(17)	3.29	14.66(2.69)	104.0
		14	187(6)	2.90	13.45(1.73)	71.9
60	0	7	213(3)	2.75	0.00	0.0
		14	209(4)	2.64	0.00	0.0
	4	7	119(37)	3.71	28.28(8.93)	237.7
		14	163(12)	3.08	12.72(1.73)	77.9

[a] Values = $\bar{x}(\pm SEM)$, n = 3 replicate cultures.
[b] Initial pH = 6.0.

sociated with aflatoxin synthesis. Instead, the genetic alteration appeared to involve a general locus associated with some aspect of the mold's primary metabolism that impacted on developmental processes.

In the current study, the lack of caffeine-dependent aflatoxin production in GMS and subsequent restoration by supplementation with a source of amino acids points to involvement of amino acid metabolism in toxin production by the mutant strains. The requirement for a source of amino acids did not relieve an auxotrophic state since the mold grew over the same range of caffeine concentrations in supplemented and unsupplemented GMS. It does not appear that amino acids were simply stimulating a low level of aflatoxin synthesis by BCR1 in GMS. If that were the case, then equivalent levels of aflatoxin production would be expected in the caffeine-containing and caffeine-free cultures. Further, the wild type strain (NRRL 2999) accumulates significant amounts of aflatoxins in GMS (Buchanan and Lewis, 1984b). Accordingly, the results suggested that the caffeine-dependent nature of aflatoxin synthesis in BCR1 involved a specific interaction between the genetic alteration and some aspect of amino acid metabolism.

Table 5—Effect of individual amino acids on aflatoxin production by A. parasiticus *BCRI cultured for 10 days in GMS with and without caffeine*[a]

Amino acid[b]	Caffeine (mg/mL)	Mycelium dry wt (mg)	pH[c]	Aflatoxin culture (μg)	Aflatoxin Mycelium (ng/mg)
None	0	137(7)	2.66	0.00	0.0
	4	182(6)	2.48	0.07(0.01)	0.4
Alanine	0	181(4)	7.80	0.00	0.0
	4	136(5)	7.67	9.44(1.70)	69.5
Arginine	0	174(18)	3.81	0.00	0.0
	4	248(14)	5.76	3.69(0.74)	14.9
Asparagine	0	195(9)	7.90	0.52(0.24)	2.7
	4	185(12)	7.36	3.33(0.54)	18.0
Aspartic	0	153(6)	8.34	0.15(0.09)	1.0
Acid	4	191(4)	6.80	0.66(0.31)	3.5
Glutamic	0	146(5)	8.26	0.24(0.16)	1.6
Acid	4	145(6)	6.72	1.87(0.42)	12.9
Glutamine	0	217(2)	7.97	0.00	0.0
	4	182(1)	7.63	0.00	0.0
Glycine	0	222(3)	8.05	0.00	0.0
	4	146(7)	7.67	0.00	0.0
Isoleucine	0	214(6)	3.12	0.12(0.12)	0.6
	4	225(15)	3.13	1.23(0.26)	5.5
Methionine	0	224(4)	2.91	0.00	0.0
	4	181(10)	3.00	7.23(1.75)	39.9
Ornithine	0	178(4)	2.63	0.00	0.0
	4	106(4)	3.24	1.56(0.76)	14.7
Proline	0	202(6)	7.68	1.21(0.39)	6.0
	4	155(4)	7.64	11.32(3.25)	73.1
Serine	0	199(1)	7.79	0.45(0.35)	2.3
	4	159(15)	7.28	1.23(0.26)	7.7
Valine	0	303(11)	3.27	0.00	0.0
	4	256(11)	3.50	1.57(0.73)	6.1

[a] Values = $\bar{x}(\pm SEM)$, n = 4 replicate cultures, except for control where n = 16 replicate cultures.
[b] Amino acids added to GMS at level of 20 mg/mL.
[c] Initial pH = 6.0.

Stimulation of aflatoxin production in wild type strains by amino acids has been observed. Several investigators (Naik et al., 1970; Reddy et al., 1979; Payne and Hagler, 1983; Sahay, 1983) reported that when wild type strains were cultured in chemically defined media containing ammonium salts as a nitrogen source, supplementation with asparagine, proline and alanine strongly stimulated aflatoxin synthesis, with lesser effects noted for aspartic acid, methionine, glycine and glutamic acid. Interestingly, a number of these amino acids, including alanine, asparagine, proline and methionine, were among the amino acids that most strongly restored caffeine-dependent aflatoxin production in the caffeine-resistant mutant, suggesting that caffeine-resistant mutant such as BCR1 might be useful in elucidating how various amino acids stimulated aflatoxin synthesis in wild type strains.

Reddy et al. (1979) and Sahay (1983) suggested that the ability of specific amino acids to stimulate aflatoxin production in wild type strains may involve their action in allowing the mold to better produce and/or accumulate catabolites (e.g., pyruvate) that serve as precursors for aflatoxin biosynthesis. It is possible that a similar explanation may account for the dependency of aflatoxin production by BCR1 on caffeine and amino acid supplementation. In the absence of both caffeine and specific amino acids, the caffeine-resistant mutant was incapable of accumulating and subsequently diverting into the aflatoxin biosynthetic pathway sufficient amounts of primary metabolites that served as precursors for aflatoxins. This could be due to the mutant having decreased glycolytic activity needed for supplying the precursors at the same time it had elevated activity in competing pathways (e.g., tricarboxylic acid cycle) that "drained off" the precursors before they could be diverted into aflatoxin synthesis. In the presence of an amino acid supplement only, there was increased availability of precursors but they were still largely diverted to competing pathways. Alternatively, in the presence of caffeine only, there was a decrease in the activities of competing pathways, but the level of precursor accumulation was still insufficient to allow abundant aflatoxin synthesis. Only when both caffeine and an ap-

propriate amino acid source were available could sufficient levels of precursor accumulation occur such that a significant portion was shunted into aflatoxin production. Additional studies with BCR1 that will be reported separately have indicated that caffeine does not directly affect aflatoxin synthesis, but instead acts by altering some aspect of primary metabolism. However, additional studies will be needed to determine if the caffeine-dependent, amino acid-dependent nature of aflatoxin synthesis in caffeine-resistant *A. parasiticus* involves a mechanism similar to that hypothesized above.

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